A Molecular Switch for Photoperiod Responsiveness in Mammals

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Report

A Molecular Switch for Photoperiod Responsiveness in Mammals

Hugues Dardente,[1,*] Cathy A. Wyse,[1] Mike J. Birnie,[1] Sandrine M. Dupré,[2] Andrew S.I. Loudon,[2] Gerald A. Lincoln,[3] and David G. Hazlerigg[1,*]
[1]Institute of Biological and Environmental Sciences, Zoology Building, Tillydrone Avenue, University of Aberdeen, Aberdeen AB24 2TZ, UK
[2]Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
[3]Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4SB, UK

Summary

Seasonal synchronization based on day length (photoperiod) allows organisms to anticipate environmental change. Photoperiodic decoding relies on circadian clocks, but the underlying molecular pathways have remained elusive [1]. In mammals and birds, photoperiodic responses depend crucially on expression of thyrotrophin β subunit RNA (*TSH*β) in the pars tuberalis (PT) of the pituitary gland [2–4]. Now, using our well-characterized Soay sheep model [2], we describe a molecular switch governing TSHβ transcription through the circadian clock. Central to this is a conserved D element in the *TSH*β promoter, controlled by the circadian transcription factor thyrotroph embryonic factor (*Tef*). In the PT, long-day exposure rapidly induces expression of the coactivator eyes absent 3 (*Eya3*), which synergizes with *Tef* to maximize *TSH*β transcription. The pineal hormone melatonin, secreted nocturnally, sets the phase of rhythmic *Eya3* expression in the PT to peak 12 hr after nightfall. Additionally, nocturnal melatonin levels directly suppress *Eya3* expression. Together, these effects form a switch triggering a strong morning peak of *Eya3* expression under long days. Species variability in the *TSH*β D element influences sensitivity to TEF, reflecting species variability in photoperiodic responsiveness. Our findings define a molecular pathway linking the circadian clock to the evolution of seasonal timing in mammals.

Results and Discussion

Circadian and Photoperiodic Influences on Pars Tuberalis *TSH*β Expression

Recently it has become clear that the pars tuberalis (PT) region of the anterior pituitary gland is a master controller of seasonal biology in mammals and birds [5]. Cells in the PT produce thyroid-stimulating hormone (TSH, also known as thyrotrophin) at levels that are increased by exposure to long days and suppressed by short days [2–4]. This photoperiodic control is mediated through changes in TSH β subunit RNA (*TSH*β) expression. PT-derived TSH acts by a retrograde mechanism on TSH receptor-expressing cells in the neighboring basal hypothalamus, which are the principal sites of type 2 thyroid hormone deiodinase (*Dio2*) expression in the hypothalamus [6]. This enzyme is a gatekeeper for the effects of thyroid hormone in the hypothalamus, controlling the availability of the active form of thyroid hormone, triiodothyronine, and dictating the expression of summer phenotypes [7, 8].

A unique feature of mammalian seasonal biology [2–5] is that these effects of day length on PT function depend on the pineal hormone melatonin, which acts via a high density of melatonin receptors localized in the PT [9, 10]. Melatonin is a circadian signal, with a nocturnal waveform proportional to the length of the night, and consequently, in the PT, melatonin controls the rhythmical expression of multiple transcription factors implicated in circadian function [11–15]. Hence, we hypothesized that photoperiodic effects on *TSH*β expression in the PT are initiated through melatonin's effects on circadian transcription factors in this tissue.

Circadian gene expression depends on three main classes of canonical circadian response element: E boxes (CANNTG), D elements (RTTAYGTAAY), and retinoid-related response elements (ROREs; WAWNTRGGTCA), each of which are regulated by cognate transcription factors [16]. Examination of the *TSH*β promoter revealed a highly conserved D element located a short distance from the transcription start site (Figure 1A; see also Figure S1A available online), potentially sensitive to the PAR-bZIP family members thyrotroph embryonic factor (TEF), D element-binding protein (DBP), and hepatic leukemia factor (HLF), which are considered important outputs of the circadian clock [17]. In luciferase reporter assays, using −3 kb of the sheep *TSH*β promoter (*TSH*β-luc), we observed transcriptional sensitivity to each of these factors (Figure 1B), and consistent with the original assignment of TEF as a transactivator of the *TSH*β promoter [18], the order of potency of these effects was TEF > HLF > DBP. Moreover, in electrophoretic mobility shift assays, TEF bound to a 35 bp oligonucleotide centered on the *TSH*β D box more strongly than either HLF or DBP (Figure 1C). Although several E boxes are also present in the proximal 3 kb of the *TSH*β promoter region, these are poorly conserved between species and do not respond to the core circadian transcription factors CLOCK and BMAL1 in the sheep (Figure S1B). No consensus RORE could be found.

We next considered whether *Tef* gene expression is photoperiodically regulated in the PT and, if so, whether these changes precede photoperiodic induction of *TSH*β expression. We used a long-day induction protocol developed previously to study seasonal neuroendocrine changes in Soay sheep [19]. This involves acclimating sheep to short photoperiod (SP, 8 hr light/16 hr dark) and then switching them to long photoperiod (LP, 16 hr light/8 hr dark) by delaying lights off by 8 hr (Figure 1D). This acutely shortens the melatonin signal, delaying its evening rise by 8 hr [19]. Stimulation of increased TSHβ immunoreactivity in the PT, hypothalamic *Dio2* expression, and plasma prolactin levels ensues during the subsequent 15 days (Figures S1C–S1E). The expression of *TSH*β RNA increases rapidly ($p < 0.001$ by two-way analysis of variance [ANOVA]), so that levels across the 24 hr cycle (area-under-the-curve estimate) are some 2.5-fold above baseline by the third day of LP (LP3), and nearly 6-fold increased by

*Correspondence: h.dardente@abdn.ac.uk (H.D.), d.hazlerigg@abdn.ac.uk (D.G.H.)

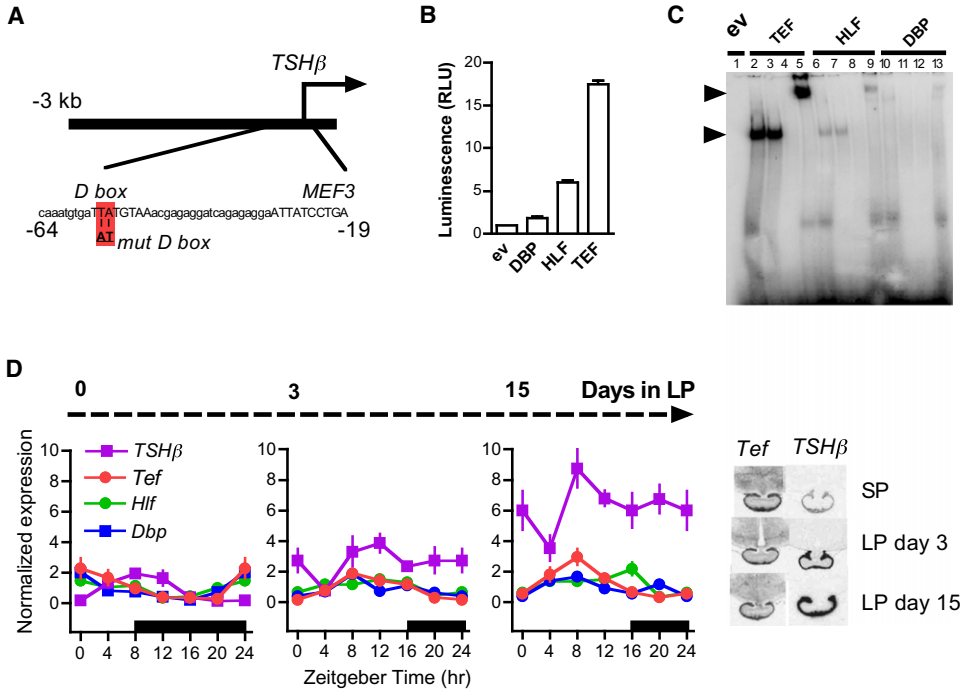

Figure 1. Pars Tuberalis Expression of *TSH*β Is Regulated by TEF and Photoperiod

(A) The *TSH*β promoter harbors conserved D and MEF3 elements; base substitutions used for loss-of-function mutation of the D element (mut D box) are shown.

(B) Luciferase assay performed in COS7 cells demonstrating the transactivating effects of expression vectors for DBP, HLF, and TEF or an empty vector (ev) on a *TSH*β promoter-reporter construct (*TSH*β-luc).

(C) Electrophoretic mobility shift assays demonstrating binding of TEF, and to a lesser extent HLF, to a 35 bp oligo centered on the *TSH*β D element. Nuclear extracts from COS7 cells transfected with an empty vector (ev) or Myc-tagged TEF, HLF, or DBP were incubated with ^{32}P-labeled oligo probe (lanes 1, 2, 6, and 10), probe plus an excess of unlabeled mut D box oligo (lanes 3, 7, and 11), probe plus excess of unlabeled wild-type oligo (lanes 4, 8, and 12), or probe plus anti-Myc antibody (lanes 5, 9, and 13). The lower and upper arrowheads indicate shifted and supershifted complexes, respectively.

(D) Photoperiodic induction of *TSH*β and PAR-bZIP factor gene expression in the pars tuberalis (PT). Soay sheep acclimated to 8 hr light per day were transferred to 16 hr light per day (LP) by acutely delaying lights off. Tissue was collected at 4 hr intervals throughout 24 hr on the 3rd and 15th day following this light manipulation and in SP control animals (0 days in LP). The black horizontal bar in each graph indicates when lights were off during each sampling period. Data are mean ± standard error of the mean (SEM) of n = 3 animals per sampling point. Representative images showing peak expression levels of *Tef* and *TSH*β in each of the sampling periods are shown at right. Further analysis of *TSH*β transcriptional control and photoperiodic effects on rhythmical gene expression in the PT can be found in Figure S1.

LP15 (Figure 1D). Over the same period, no significant increase in the expression of *Tef*, *Dbp*, or *Hlf* was observed, although the phase of peak expression of all of these genes was delayed by approximately 8 hr ($p < 0.01$ by two-way ANOVA for photoperiod × zeitgeber time [ZT] interaction in all cases). This effect was also seen across a wider selection of genes associated with circadian function (Figure S1F), reflecting the importance of the timing of melatonin onset for the phasing of rhythmical gene expression in the PT [12, 13, 19].

Eya3 Is a Photoperiodically Induced Coactivator for TEF-Induced *TSH*β Transcription

The absence of a photoperiodic effect on the amplitude of *Tef* expression in the PT suggested to us that additional factors interact with TEF to control *TSH*β expression in this tissue. Two recent studies highlight the transcriptional coactivator eyes absent 3 (*Eya3*) as being strongly induced by increasing photoperiod in the PT [4, 20]. *Eya3* is of particular interest because it is a member of a developmental regulatory network including the *Pax* and sine oculis (*Six*) gene families believed to be critical for the formation of structures including the eyes and pineal and pituitary glands [21–23]. Intriguingly, in the PT of the Japanese quail, expression of *Eya3* rises in parallel with that of *TSH*β following exposure to long days [4], hinting at a role early in the photoperiodic induction process.

We therefore compared the daily profiles of *Eya3* and *TSH*β expression in the sheep PT under SP and at LP3 and LP15 (Figure 2A). Under all three conditions, *Eya3* showed a transient peak in expression approximately 12 hr following dark onset, with an amplitude that increased 3-fold by LP15 ($p < 0.001$ by two-way ANOVA for photoperiod × ZT interaction). Under LP, peak *Eya3* expression preceded that for TSHβ by some 4 hr, supporting the concept that EYA3 may be involved in transcriptional activation of *TSH*β.

EYA3 is thought to act by dimerizing with SIX-family proteins, forming a transcriptional coactivator complex [21–23]. We therefore analyzed tissue expression patterns for the sheep orthologs of various *Six* family members by reverse transcriptase-polymerase chain reaction and in situ hybridization (Figures S2A and S2B). Whereas *Six1* had a highly PT-enriched pattern of expression, other members were ubiquitously expressed (*Six4*), enriched in neighboring hypothalamic sites (*Six6*), or undetectable (*Six2*). Although *Six1* expression in the PT was not rhythmic (Figure S2B), it increased slightly with LP exposure, so that mean levels were some 50% higher at LP15 than in SP controls ($p < 0.05$).

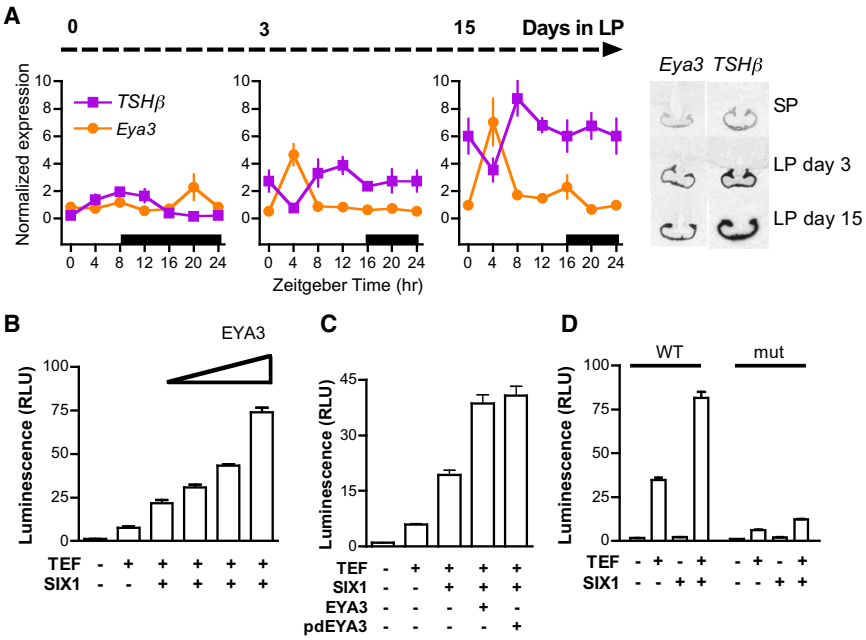

Figure 2. Photoperiodic Induction of *Eya3* and Its Potentiating Effect on *TSH*β Expression

(A) Temporal regulation of *Eya3* and *TSH*β expression in the PT during the same photoperiodic induction experiment described in Figure 1D, presented identically. Data are mean ± SEM of n = 3 animals.

(B) TEF-induced transactivation of the *TSH*β-luc reporter is enhanced by addition of SIX1, and this effect is further potentiated by addition of EYA3 (25–100 ng).

(C) A phosphatase-dead EYA3 mutant (pdEYA3, D263A) also potentiates the TEF/SIX1 response.

(D) TEF action and TEF/SIX1 synergism are lost after mutation of the D element (mut D box; see Figure 1A). Further details of *Six* expression in the PT and *Eya3* transcriptional control can be found in Figure S2.

Returning to the *TSH*β promoter, we identified a putative MEF3 site, predicted to mediate EYA3/SIX1 actions [24, 25], downstream of the D element (see Figure 1A; Figure S1A); this is also conserved across mammals. In further reporter assays, SIX1 had a mild potentiating effect on the actions of TEF, with the *TSH*β-luc response enhanced 2.5-fold. Strikingly, cotransfection of EYA3 (25–100 ng) with SIX1 and TEF (25 ng each) caused a dramatic synergistic enhancement of reporter activity, increasing the response by an order of magnitude compared to that seen for TEF alone (Figure 2B). This effect of EYA3 appeared to be independent of its reported tyrosine phosphatase activity, because a phosphatase-dead EYA3 (pdEYA3) in which a single point mutation disrupts the catalytic domain (D263A; see [25]) showed a potency similar to wild-type (WT) EYA3 for potentiating TEF/SIX1 action (Figure 2C). No independent effects of EYA3 or SIX1 on *TSH*β-luc activity were observed (Figure S2C).

Mutations of the putative MEF3 site present in the *TSH*β promoter had a negligible effect on the combined TEF/SIX1 response (Figure S2D), supporting the concept that EYA3 and SIX1 act as coactivators of TEF rather than as an independently transcriptionally active heterodimer. In line with this, we found that mutating the D element to destroy the palindromic half-site organization suppressed both the direct effects of TEF and the potentiating effects of SIX1 (Figure 2D), suggesting that this element is crucial for photoperiodic regulation of *TSH*β expression.

Circadian and Melatonin-Dependent Control of *Eya3* Transcription

We next considered the mechanism whereby *Eya3* expression is increased by the transfer to LP. Exploration of the *Eya3* promoter demonstrated that it is controlled through three conserved E boxes sensitive to CLOCK and BMAL1 and through a conserved D element sensitive to TEF and SIX/EYA3, all located with 500 bp of the transcription start site (Figures 3A–3C; Figure S2E). This promoter organization accounts for the rhythmical expression of *Eya3*, which, like other rhythmic genes in the PT, is controlled by melatonin [14]. Melatonin onset at lights off is the major phase-resetting signal in this tissue, so that long days generate waves of gene expression in the PT peaking later relative to dawn than do short days [14] (see also Figure S1F). We have shown previously that, in addition to setting the phase of gene expression rhythms in the PT, melatonin directly suppresses the expression of a range of E box-controlled genes [15]. Similarly, melatonin implants given to sheep acclimated to LP and then exposed to constant light suppress *Eya3* expression (Figure 3D). These effects probably stem from melatonin's suppression of cAMP signaling, because derepression of cAMP-dependent pathways following melatonin withdrawal at dawn is critical for the morning peak of *Per1* expression seen in the PT [13]. We are currently investigating possible cAMP-dependent regulation of the *Eya3* promoter.

These two classes of effect of melatonin on *Eya3* expression, phase synchronization and direct suppression, lead to a molecular model linking the circadian system to the photoperiodic response (Figure 4). The model postulates that the phase of *Eya3* expression in the PT is critical for determining whether a strong peak does or does not occur. Independent of day length, *Eya3* peaks some 12 hr after dark and melatonin onset. This means that under short days, the peak occurs during the night, while the melatonin level is high and exerting a suppressive effect, and so the peak is small. Contrastingly, under long days, the *Eya3* peak occurs the following morning when the melatonin level is minimal, and so the peak is large. This classic "external coincidence timer" mechanism, in which a circadian oscillation interacts with a light-dependent stimulus [1], limits EYA3/TEF synergism to long days, controlling the onset of a summer phenotype.

In addition to this primarily circadian photoperiodic induction process, we suggest that the further amplification of the *Eya3* peak, seen between LP3 and LP10, may also be due to amplification through transcriptional autoregulation at the *Eya3* promoter via TEF/EYA3/SIX effects through the D element (see Figure 3C). The *Tef* gene also includes a promoter sensitive to E boxes and D elements in combination (Figure S3), and recent work has identified *Tef*-driven, D element-mediated effects as crucial for light entrainment of the E box-driven *period* gene rhythmicity at the core of the zebrafish circadian

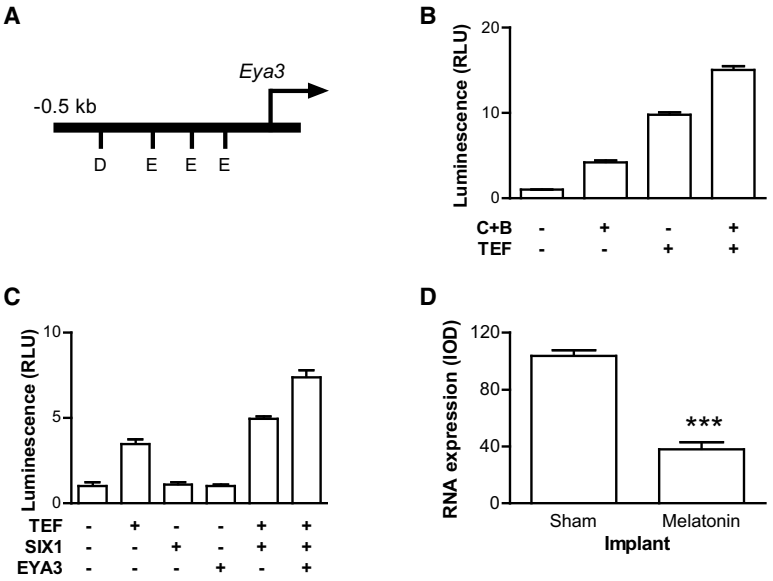

Figure 3. *Eya3* Is Transcriptionally Controlled through E Boxes and D Elements

(A) The proximal region of the *Eya3* promoter harbors one conserved D element and three conserved E boxes.

(B) CLOCK and BMAL1 (C+B) and TEF have additive effects on a 0.5 kb *Eya3*-luc reporter in COS7 cells.

(C) SIX1 and EYA3 potentiate the effects of TEF on *Eya3*-luc activity.

(D) *Eya3* expression is suppressed in LP-acclimated sheep exposed to constant light prior to melatonin administration 16 hr after lights on and then sacrificed 3.5 hr later. Data are mean ± SEM from n = 6 animals in each group. ***p < 0.001, significantly reduced expression relative to sham-injected control animals by independent t test. Evidence for combined E box/D element control of *Tef* expression is given in Figure S3.

clock [26]. Hence, E box/D element interactions are a recurrent feature of light-modulated circadian circuits.

Variation in *TSH*β D Element Function Is Correlated with Photoperiodic Sensitivity

Our observations implicate the proximal D element in the *TSH*β promoter as a convergence point in the transcriptional network controlling the photoperiodic response. Across mammals, there is pronounced variability in the strength of photoperiodic influences on seasonal physiology [27]. We therefore explored the possibility that species variation in photoperiodic sensitivity might be due to sequence variability in the *TSH*β D element (see Figure S1). Like sheep, melatonin-proficient strains of mice show photoperiodic *TSH*β expression [3] and a G→A substitution at position 5 of the core D element (Figure 5); variability at positions 3 and 4 is seen among primates and in pigs, among which the expression of seasonal photoperiodism varies considerably. We therefore mutated the sheep *TSH*β-luc reporter to contain human-, marmoset-, pig-, or mouse-like D elements and assessed sensitivity to TEF activation (Figure 5). Remarkably, this produced a high degree of variability in TEF response, with the maximal effect being seen with the mouse-like form, which is a perfect eight-base palindrome, followed by the sheep-like variant. The weakest response occurred with the D element motifs from the marmoset and the domestic pig: species that are not overtly photoperiodic, because of their natural ecology or artificial selection during domestication, respectively [27–29]. This contrasts with the case of the rhesus macaque, an example of a seasonally photoperiodic primate [30], carrying a mouse-like *TSH*β D element. Hence, along with variability in melatonin signal production and TSH receptor signaling [31], *TSH*β promoter organization is a likely contributor to photoperiodic sensitivity. An intermediate level of response was seen for the human-like form, suggestive of attenuated photoperiodic sensitivity through the TEF-dependent pathway. In this regard, it is noteworthy that the seasonal melatonin response pathway has been implicated in seasonal affective disorder [28], and it will be of interest to explore genetic variability in the human *TSH*β promoter locus.

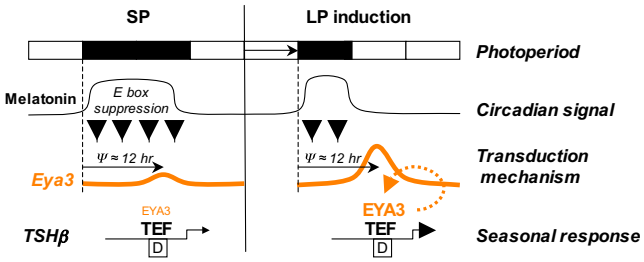

Figure 4. Model for Photoperiodic Induction of *Eya3* Expression in the Pars Tuberalis

See text for details.

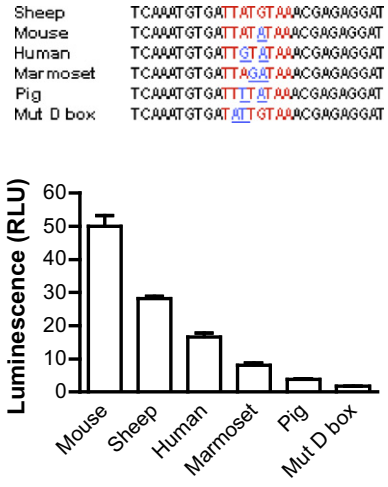

Figure 5. Comparative Differences in *TSH*β D Element Function in Relation to Photoperiodic Sensitivity

Upper panel: comparison of the core sequence in the *TSH*β D element across mammals reveals several variant forms exemplified by the species shown. Lower panel: COS7 cell reporter assay of TEF responsiveness, using the sheep *TSH*β-luc reporter mutated to give the variant forms shown in the upper panel. Data are shown as relative luciferase units (RLU), normalized to control values in the absence of TEF, and are mean ± SEM of triplicate observations.

Conclusion

In conclusion, we have defined a pathway linking day length to seasonal biology via a circadian, melatonin-dependent pathway. This pathway is the mammalian form of an ancestral photoperiodic timer, and we predict that a similar clock-controlled, *Tef*/*Six1*/*Eya3*-dependent mechanism will emerge as the conserved driver of seasonal change in birds and other vertebrate groups, even though the importance of melatonin for this process appears peculiarly mammalian [5]. Within the mammalian photoperiodic system, circadian properties are seen at multiple levels, notably the suprachiasmatic nucleus and pineal gland [1], as well as in the melatonin-dependent PT. What makes the PT remarkable is that it behaves like a circadianly controlled photoperiodic switch—a derived function that appears to depend crucially on promoter organization of clock-controlled genes lying upstream in the TSH-*Dio2* pathway. Synthetic biology approaches highlight the capacity for combinations of the basic classes of circadianly controlled promoter elements to dictate phase of expression of rhythmical gene expression [16]. We believe that nature's tinkering with this principle has been the key to the evolution of light entrainment and photoperiodic response pathways.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.cub.2010.10.048.

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